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Development associated profiling of chitinase and microRNA of *Helicoverpa armigera* identified chitinase repressive microRNA

Neema Agrawal*, Bindiya Sachdev*, Janneth Rodrigues, K. Sowjanya Sree & Raj K. Bhatnagar

International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, P.O. Box 10504, New Delhi-110067, India.

* These authors contributed equally to this work.

Expression of chitinase is developmentally regulated in insects in consonance with their molting process. During the larval-larval metamorphosis in *Helicoverpa armigera*, chitinase gene expression varies from high to negligible. In the five-day metamorphic course of fifth-instar larvae, chitinase transcript is least abundant on third day and maximal on fifth day. MicroRNA library prepared from these highest and lowest chitinase-expressing larval stages resulted in isolation of several miRNAs. *In silico* analysis of sequenced miRNAs revealed three miRNAs having sequence similarity to 3′UTR of chitinase. Gene-targeted specific action of these miRNAs, was investigated by luciferase reporter having 3′UTR of chitinase. Only one of three miRNAs, *miR-24*, inhibited luciferase expression. Further, a day-wise *in vivo* quantification of *miR-24* in fifth-instar larvae revealed a negative correlation with corresponding chitinase transcript abundance. The force-feeding of synthetic *miR-24* induced significant morphological aberrations accompanied with arrest of molting. These *miR-24* force-fed larvae revealed significantly reduced chitinase transcript abundance.

MicroRNAs (miRNAs) are small non-coding endogenous RNAs (ca. 22 nucleotides) derived from much longer pre-primary transcripts[1]. The miRNAs represent a major class of post-transcriptional regulatory molecules, which play a key role in a wide diversity of biological processes such as development, metabolism and apoptosis in eukaryotic organisms[2–4]. They control the expression of target genes through binding to complementary target sites in mRNA targets[5,6,1]. In animals, these sites typically have imperfect complementarity to the miRNA. The ability to target imperfectly complementary target sites, along with other miRNAs in the genome, has led to the prediction of thousands of genes which are miRNA regulated[7,8]. However, despite the apparent abundance of potential miRNA/3′UTR target interactions, only few animal miRNA/target pairs have been validated till date in a physiologically relevant context[9,10].

An array of diverse miRNAs are constitutively expressed whereas a few others show temporal and tissue-specific expression patterns[11]. A number of miRNAs have been isolated and annotated from several eukaryotic organisms including insects. Nearly 21,264 miRNAs were deposited in the latest miRBase database release 19.0 in August 2012. Nearly 3457 insect miRNAs have been identified and these mainly restrict to Diptera (*Drosophila* sp., *Anopheles gambiae*, *Aedes aegypti*, *Culex quinquefasciatus*), Hymenoptera (*Nasonia* sp., *Apis mellifera*), Coleoptera (*Tribolium castaneum*), Orthoptera (*Locusta migratoria*), Lepidoptera (*Bombyx mori*, *Manduca sexta*, *Spodoptera litura*, *Helicoverpa armigera*, *Heliothis virescens*) and Homoptera (*Acyrthosiphon pisum*) (http://www.mirbase.org/). However, the targets of most of these annotated miRNAs have not been identified yet.

In *S. litura*, 58 putative miRNAs have been identified using computational methods. The temporal and spatial expression profiles of these miRNAs have been examined in different tissues and stages of development[12]. By sequencing small RNA libraries of *M. sexta* embryos, larvae, pupae and adults, 163 conserved and 13 novel miRNAs have been identified so far[13]. Homology-based predictions of lepidopteran miRNAs have been conducted in *H. armigera* and *H. virescens*[14,15]. Nevertheless, a few miRNAs that control metamorphic transition from larvae to pupae have been functionally identified in *B. mori*[16].

Extensive softwares have been developed to tentatively identify the targets of miRNAs but the identification of precise biological function targeted by miRNAs still remains a challenge. It has been estimated that the false positive rate of *in silico* predicted miRNAs is 24–70%[17]. This inability to predict and correlate miRNA controlled metabolic events, emphasizes the need for experimental validation of the predicted miRNAs, in order to identify

their specific targets. Several different approaches are being pursued to identify miRNA targets and have been recently described[18].
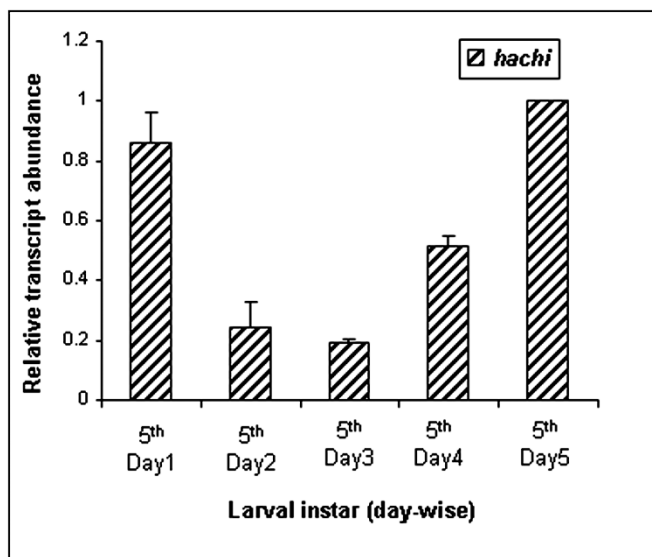
We have been working on the profiling of chitinase (EC 3.2.1.14) at various larval transition stages in *Helicoverpa armigera*[19]. In insects, chitin forms a major component of the cuticle and the peritrophic membrane of the midgut[20]. Chitinase catalyzes the degradation of chitin, a linear homopolymer of N-acetylglucosamine in a β-(1,4) linkage and plays a major role in insect metamorphosis[21]. The expression of chitinase coincides with the molting of larvae and is regulated by ecdysone[19,21].

To profile relative expression abundance of various microRNAs at various stages of chitinase expression, an miRNA library was prepared from different larval stages. These microRNAs were enriched by their ability to hybridize with 3′ untranslated region (UTR) of chitinase gene. Using solution hybridization, we identified a few putative miRNAs targeting the chitinase gene in *H. armigera (hachi)*. The correlation between putative microRNAs interacting with 3′UTR of *hachi* was examined by cloning it downstream of 3′end of firefly luciferase reporter gene *(Fluc)*. The direct involvement of *miR-24* in the molting process was examined by force-feeding synthetic *miR-24* to *H. armigera* larvae. The *in vivo* levels of this miRNA at various stages of larval development corresponding to chitinase expression were quantified. These force-fed larvae showed arrested molting process with corresponding reduction of chitinase transcript abundance.

Results

Temporal expression of *H. armigera* chitinase (*hachi*). The molting stage of 5th instar larvae of *H. armigera* is spread over 5 days followed by pupation. The relative levels of *hachi* transcript in different days of 5th instar larvae were analyzed by Real-time qPCR (Figure 1).

A varying pattern of *hachi* expression was observed in 1–5 day old, 5th instar larvae (Figure 1). High chitinase level is required for dissolution of exoskeleton and permits expansion of larvae mass. Chitinase transcript was highly abundant on the first and fifth day

of 5th instar i.e. immediately upon molting and close to pre-pupa stage. However, detectable *hachi* transcript was present during inter-molting stages on second, third and fourth days. Nearly, 2-folds increase in *hachi* transcript was observed on fifth day (last day) as compared to fourth day (Figure 1). The observed pattern of chitinase expression coincides with reported levels of chitinase enzyme[19]. Such pattern reflects the developmental necessity of chitinase for insect to molt.

Isolation and functional validation of chitinase-specific miRNAs. Chitinase-specific miRNAs were isolated from *H. armigera* midgut tissue using Solution hybridization technique. The hybridized small RNAs were cloned into pGEM-Te vector and the recombinant colonies were initially screened using vector-specific primers. Approximately 50 insert positive colonies were sequenced and out of these 50, three colonies revealed sequences that displayed varying degree of complementarity to 3′UTR of chitinase. The three putative miRNAs viz. *miR-24*, *miR-2* and *miR-131* were detected which displayed >65% sequence similarity to the 3′UTR of *hachi* (Figure 2; Table 1).

The potential miRNA/3′UTR target interactions were studied by conducting luciferase assays. The 3′UTR of *hachi* was cloned downstream to 3′end of firefly luciferase gene *(Fluc)* in pIB/V5-His Topo vector to generate firefly luciferase reporter construct. Co-transfection of this luciferase reporter construct along with pIB-*Rluc* vector and synthetic *miR-24* into *Sf*21 cells resulted in inhibition of luciferase activity (Figure 3). Dose-dependent reduction in luciferase activity was observed with nearly five-folds reduction in luminescence upon co-transfection with 200 nM of synthetic *miR-24* as compared to controls. In contrast, other miRNAs that displayed sequence similarity to 3′UTR of *hachi* viz. *miR-2* and *miR-131* did not inhibit luciferase activity (Figure 3).

***In vivo* levels of *miR-24* in *H. armigera* larvae.** Having established the pattern of chitinase gene expression at different days during molting, we estimated levels of *miR-24* at corresponding stages. The quantitative analysis of *miR-24* in different days of 5th instar larvae of *H. armigera* was analyzed by Real-time qPCR (Figure 4).

Figure 1 | Transcript abundance of *H. armigera* chitinase *(hachi)*. The relative abundance of *hachi* transcript in 5th instar larvae of *H. armigera* (1–5 day old) was determined by Real-time qPCR. Total RNA isolated from the midgut tissue was used as template and normalised to internal control, *β-actin*. Expression levels of *hachi* were measured using Comparative C_T method. A non-template control was run with every experiment. Representative data (mean ±S.D.) from three independent experiments is shown.

Figure 2 | Nucleotide sequence of 3′UTR of *H. armigera* chitinase. A 1057 bp fragment encoding the 3′UTR of *hachi* has been cloned in pGEM-Te vector and sequenced. The nucleotide sequence of 3′UTR corresponding to *miR-2*, *miR-24* and *miR-131* (miRBase) is shown. The bases in black are completely identical while those in red are mismatches.

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Table 1 | Sequence and percentage similarity of putative chitinase-specific miRNAs isolated from *H. armigera* midgut tissue

miRNA	Sequence of putative miRNAs	% sequence similarity with *hachi* 3′UTR	Synthetic miRNA sequence
miR-2	5′-TGCGCAAATGAGGAA-3′	68.75	Sense: GCAAUUAGCAAAUGAGGGUdTdT Antisense: UUCCUCAUUUGCGCAAUGCdTdT
miR-24	5′-ATCCTCAGTTCA-3′	75	Sense: CGGGUUGCAUCUCAGUUCAdTdT Antisense: UGAACUGAGGAUCAACCCGdTdT
miR-131	5′-GTTAGTGAA-3′	89	Sense: GAUGUUUUAGGUUACUGAAdTdT Antisense: UUCAGUAACCUAAAACAUCdTdT

QuantiMir cDNA was prepared from midgut total RNA and normalised to *β-actin* which served as internal control. The levels of *miR-24* increased steadily from day1, peaking at day 3 and reduced subsequently upto day 5. The transcript level of *miR-24* was nearly 3-folds less on the first and fifth day of 5th instar as compared to third day. Nearly, 5-folds increase in *miR-24* transcript was observed on the third day as compared to fifth day (Figure 4).

An overall inverse pattern of *miR-24* expression was observed in relation to chitinase transcript levels. Thus, the observed inverse relationship of *miR-24* and chitinase together with sequence similarity of *miR-24* to 3′UTR of *hachi* suggested that *miR-24* indeed is involved in the regulation of chitinase.

**Phenotypic effect of synthetic *miR-24* on *H. armigera* larvae.**
Comparative data on the relative abundance of *miR-24* and chitinase clearly revealed inverse relationship suggesting that high *miR-24* levels would block molting process. To investigate further, we administered synthetic *miR-24* to the 4th instar two-day old larvae. Upon ingestion of synthetic *miR-24* (0.02 nmoles), the larvae ceased to feed and could not molt further (Figure 5; Table 2). The relative expression level of *hachi* was monitored in both *miR-24* fed and control larvae by Real-Time qPCR (Figure 6). Nearly, 2-folds reduction in *hachi* transcript was observed in *miR-24* fed larvae after 24 hrs as compared to controls (Figure 6). After 48 hrs, the larvae did not molt, were shrunken and eventually died (Figure 5; Table 2).

Discussion

The temporally regulated expression of chitinase gene in 5th instar larvae of *H. armigera* focussed our investigation towards its regulation and prompted to explore the role of miRNAs. Instead of the standard protocol of *in silico* miRNA prediction followed by their experimental validation for target identification, a reverse *in vitro* analysis was carried out to identify the miRNAs targeting chitinase mRNA. The strategy employed in the present investigation facilitated the identification of microRNAs that displayed sequence similarity to 3′UTR of *hachi*. As in animal microRNA targets, the sequence similarity was imperfect that varied from 69–85%[22].

A comparative analysis of all these tentatively identified miRNAs revealed varying degree of sequence similarity with 3′UTR of chitinase. The *miR-2*, *miR-131* and *miR-24* displayed 69, 89 and 75% sequence similarity respectively at different regions of 3′UTR. Of these, *miR-24* displayed perfect match at 4-12 position and may be representing the seed region. The percentage sequence similarity did not correlate with the observed down regulation of chitinase indicating that mere sequence identity cannot be the prime criteria for target identification[23].

Several studies have shown that only a few *in silico* predicted miRNAs eventually are validated by *in vivo* experimentation[24,25,18]. To identify the functional miRNAs, all three miRNAs, *miR-2*, *miR-131* and *miR-24* were examined further for their functional interaction with 3′UTR of *hachi* by employing dual luciferase assay system. The assay employed pIB/V5-His Topo vector with 3′UTR
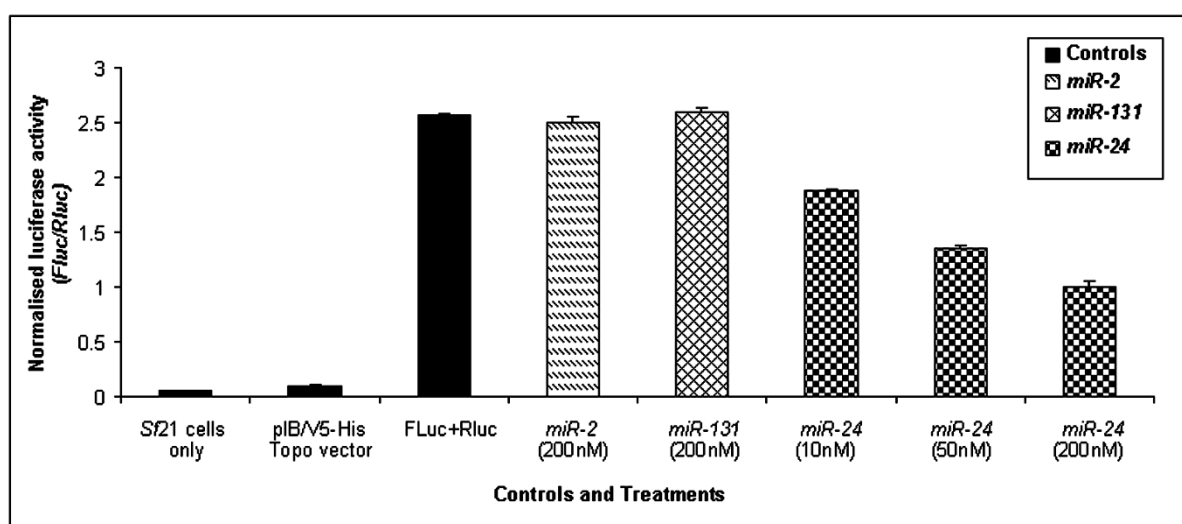
Figure 3 | **Functional validation of chitinase-specific miRNAs using luciferase-based reporter assay in *Sf*21 cells.** The 3′UTR of *hachi* was cloned downstream to firefly luciferase *(Fluc)* in pIB vector. *Sf*21 cells were co-transfected with recombinant pIB-*Fluc*-3′UTR*hachi*, pIB-*RLuc* control vector and *miR-2* or *miR-131* or *miR-24* (10–250 nM). Dual-luciferase reporter assays were performed 48 hrs after transfection. Firefly/Renilla luciferase ratios were calculated. Histogram depicts the normalised luciferase activity from three independent experiments. The following controls were set up: Control 1: Only *Sf*21 cells; Control 2: (pIB): *Sf*21cells transfected with pIB vector alone; Control 3: (FLuc+RLuc): *Sf*21 cells were co-transfected with pIB-*Fluc*+pIB-*Rluc*. The control set of experiments were also repeated thrice in triplicates.
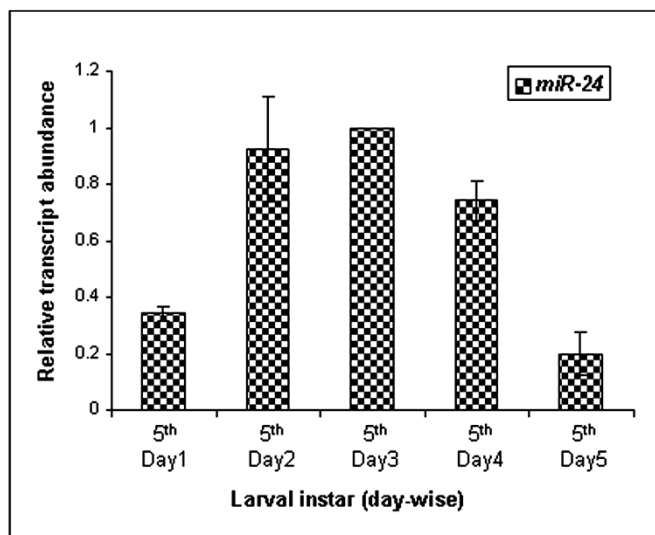
Figure 4 | Quantitation of *miR-24* in *H. armigera* larvae. The relative abundance of *miR-24* transcript was estimated in 5th instar larvae of *H. armigera* (1–5 day old) by Real-time qPCR. Total RNA was extracted from the midgut tissue and used as template. Transcripts were normalised to *β-actin* control. Expression levels of *miR-24* were measured using Comparative C_T method. A non-template control was run with every experiment. Representative data (mean ±S.D.) from three independent experiments is shown.

of *hachi* cloned after the 3′end of *Fluc*. This construct was used for assessing the effect of miRNAs individually. Co-transfection of firefly luciferase reporter plasmid with *miR-24* showed inhibition of luciferase activity in a concentration-dependent manner whereas *miR-2* and *miR-131* did not show any effect on luciferase activity. The participation of *miR-24* in regulating expression of chitinase was further substantiated by the observed low levels of *miR-24* at developmental stage which displayed highest level of chitinase transcript. In the present study, *miR-24* levels were highest at day 3 of 5th instar larva, at this stage chitinase transcript abundance was minimal. The inverse relationship in the temporal expression pattern of *miR-24* and chitinase provides clear evidence of negative regulation of chitinase gene expression by *miR-24*. The microRNAs are known to mediate their effect through target miRNA degradation or through translational repression[22]. At present it is difficult to speculate on the mechanism of action of *miR-24* on chitinase transcript abundance[22].

A direct correlation of *miR-24* regulation of chitinase expression was revealed in experiments where larvae were force-fed with *miR-24*. The *miR-24* force-fed larvae were shrunk, could not moult and also showed drastic reduction in the chitinase transcript levels depicting morphological and physiological changes *in vivo*. This suggests that *miR-24* acts as a genetic switch in the regulatory cascade of chitinase gene function[22]. Taken together, our results reveal that modulation of relative levels of *miR-24* have a direct bearing on important morphogenetic developmental process. Also, most importantly our approach of identifying microRNAs that control diverse developmental events could be applied to other tightly regulated biochemical processes.

Methods

Insect rearing, midgut dissections and RNA isolation. The culture of *H. armigera* was maintained in our insectary under controlled conditions of temperature 25°C, 70% relative humidity and a photoperiod of 14 hrs light: 12 hrs dark. The larvae were reared on a semi-synthetic artificial diet. Fifth-instar larvae (1-5 day old) were chilled on ice for 15 min and dissected to isolate the midgut tissue. The midgut was slit longitudinally, the peritrophic membrane was removed and the residual midgut contents were rinsed away with diethyl pyrocarbonate (DEPC)-treated water. The
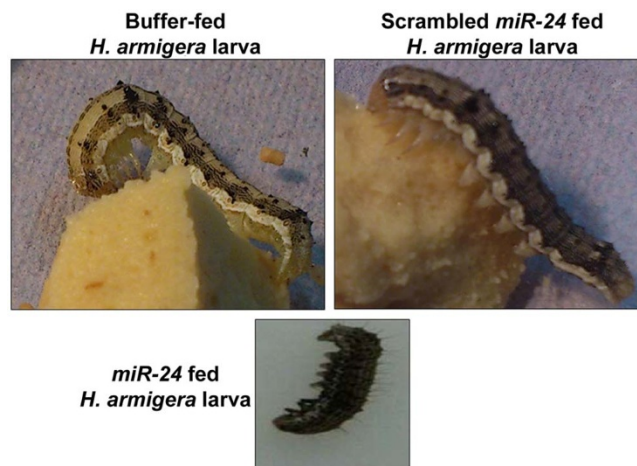
Figure 5 | Phenotypic effect of *miR-24* in *H. armigera* larvae. The *H. armigera* larvae (4th instar, two-day old) were force-fed with synthetic *miR-24* (0.02 nmoles) using a microinjector. Larvae fed with Ringer's buffer and scrambled synthetic *miR-24* served as controls. The larvae were monitored upto 48 hrs and morphological changes were observed.

midgut tissue was either used immediately or snap frozen in liquid nitrogen and then stored at −80°C until further use.

Total RNA was isolated from the midgut tissue using Trizol reagent (Invitrogen, USA) as directed by the manufacturer.

Real-Time qPCR analysis. The relative transcript abundance of chitinase was determined by Real-Time qPCR analysis using GENE-REAMix™ SYBR One Step kit (Puregene, Genetix Biotech, India) and StepOne™ Real-Time PCR System (Applied Biosystems, USA). Gene-specific primers were designed from cDNA sequence of *H. armigera* chitinase (NCBI accession no. AY325496). The chitinase transcript was amplified using primers (HelioN3 For: 5′-AGGAACTTCACAGC TCTTCG-3′ and HelioC2 Rev: 5′-CTCATAAGCCCACTGATCATG-3′). The amount of total RNA was normalised to *β-actin* transcript using primers (*β-actin* For: 5′-CAGATCATGTTTGAGACCTTCAAC-3′ and *β-actin* Rev: 5′-GA/C/ TCCATCTCC/TTGCTCGAAA/GTC-3′). Each 25 μl reaction mixture contained 2 μl of template RNA (100–500 ng/μl), 2X Gene-REAMix™ SYBR One Step, 0.25 μl of forward and reverse primer (25 μM), 0.5 μl of RiboSafe RNase Inhibitor (10 U/μl) and RNase-free water. Real-time cycling conditions included a preliminary reverse transcription at 42°C for 10 min, an initial activation step at 95°C for 10 min followed by 40 cycles each of 95°C, 15 sec; 52°C, 15 sec and 72°C, 15 sec respectively. The final step included gradual temperature increase from 60°C to 95°C at the rate of 0.3°C/ 10 sec to enable melt-curve data collection. A non-template control (NTC) was run with every assay. Reactions were set up in triplicates.

The threshold cycles (C_T) were recorded for each amplicon (*hachi* and *β-actin*) and the difference between the C_T (i.e ΔC_T) was determined. The relative abundance was calculated using Comparative C_T method using the formula $2^{-\Delta\Delta CT}$[26].

Isolation and synthesis of chitinase-specific miRNAs. *Isolation of small RNAs from midgut tissue.* Total RNA isolation and small RNA enrichment procedure were performed using *mir*Vana™ miRNA Isolation Kit (Ambion, USA) as directed by the manufacturer. Briefly, the midgut tissue was homogenized in lysis buffer. The lysate was then extracted with acid-phenol:chloroform, added ethanol to bring up the sample to 25% ethanol and fractionated to isolate the large and small RNAs using glass-fiber filter. RNA species (<200 nt) were visualized on a 15% denaturing polyacrylamide gel.

Cloning of 3′UTR of H. armigera chitinase. The full-length *H. armigera* chitinase (*hachi*) has been cloned and characterized in our laboratory using RACE technology[19]. Using plasmid DNA of 3′RACE product of *hachi* as template and gene-specific primers (HaUTR For: 5′-TGGTCATTGCACAGATACCGT-3′) and (HaUTR Rev: 5′-GGATCCACCATAATTTATTGTCTTA-3′), a 1057 bp fragment encoding 3′UTR of *hachi* was amplified. This fragment was cloned into the pGEM-Te vector (Promega, USA) and sequenced (Macrogen, Korea). The RNA transcript of the 3′UTR of *hachi* was synthesized by *in vitro* transcription using T7 RNA polymerase (MBI Fermentas, USA).

Solution Hybridization assay. The miRNA expression levels in small RNA samples were measured by solution hybridization using *mir*Vana™ miRNA Detection Kit (Ambion, USA) as directed by the manufacturer. Briefly, small RNA species and RNA transcript of 3′UTR of *hachi* were hybridized at 42°C for 12 hrs. Single-stranded RNAs were digested using RNase A/T1 solution and RNA-RNA hybrids were protected and precipitated. To clone the protected RNAs, an oligoribonucleotide

Table 2 | Effect of synthetic *miR-24* on *H. armigera* larvae (4th instar, 2 day old) after 48 hrs of force-feeding

H. armigera larvae	% mortality	Morphological changes
Buffer-fed (Control larvae)	0	Healthy and molted to 5th instar
Scrambled *miR-24* force-fed larvae (Non-specific control)	0	Healthy and molted to 5th instar
miR-24 force-fed larvae (Treated larvae)	100	Shrunken, could not molt and died

was ligated using T4 RNA ligase (MBI Fermentas, USA) and cDNA was synthesized using SuperScript III (Invitrogen, USA). The cDNA was polyG-tailed at 3′end using terminal deoxynucleotidyl transferase (Invitrogen, USA). Using primers complementary to the adaptors, the cDNA was amplified and cloned into pGEM-Te vector and sequenced (Macrogen, Korea).

miRNA synthesis. Based on the sequence of the isolated miRNAs and their sequence identity to the 3′UTR of *hachi*, double-stranded miRNAs were synthesized commercially (Dharmacon, USA). These synthetic miRNAs were used for luciferase reporter assay and force-feeding experiments.

Cell culture, transfections and luciferase assay. Full-length Firefly luciferase (*Fluc*) and Renilla luciferase (*Rluc*) cDNAs were amplified and cloned in an insect expression vector, pIB/V5-His TOPO (Invitrogen, USA) and named 'pIB-*Fluc* control vector' and 'pIB-*Rluc* control vector' respectively. The 3′UTR of *hachi* was cloned downstream to pIB-*Fluc* after the 3′end of *Fluc* in EcoRV/SacII sites. This construct was referred as 'recombinant firefly luciferase reporter vector' or 'pIB-*Fluc*-3′UTR *hachi*'.

For luciferase assays, *Sf*21 cells were grown and maintained at 27°C in BD Baculogold TNM-FH insect medium (BD Biosciences, USA) containing 10% fetal bovine serum and gentamicin. Cells were grown as a monolayer upto 70–80% confluence in T-75 flasks (Nunc, USA). Two hours before transfection, *Sf*21 cells (60–70% confluent) were scraped and approximately 4×10^5 cells/well were seeded into 6-well plates. Cells were co-transfected with pIB-*Fluc* plasmid (1 μg), pIB-*Rluc* plasmid (1 μg) and *miR-2* or *miR-131* or *miR-24* at varying concentrations (10–200 nM) using cellfectin reagent (Invitrogen, USA) in a final volume of 0.6 ml. Four hours post-transfection, serum plus medium was added to the cells and the culture plate was held stationary at 27°C for 48 hrs.

After 48 hrs, Firefly and Renilla luciferase activity was measured sequentially using Dual Luciferase assay kit (Promega, USA) as directed by the manufacturer.

Luminescence was read on a Packard LumiCount manual luminometer (Cole Parmer, USA). Firefly luciferase values were normalised to Renilla luciferase values and the luciferase levels were reported as ratio of FLuc/RLuc. This normalised luciferase activity was plotted. Each transfection was tested in three independent experiments, each performed in triplicates. Luciferase activity was measured thrice for each sample.

The following controls were set up. Only *Sf*21 cells served as first control while only *Sf*21cells transfected with pIB vector only served as second control. Both did not show luminescence. In third control, *Sf*21 cells were co-transfected with pIB-*Fluc* vector and pIB-*Rluc* vector without 3′ UTR of *hachi*. Luminescence from the test samples were normalized against the ratio of Fluc/Rluc obtained from the third control. Further, a parallel set of controls included *Sf*21 cells transfected individually with pIB-*Fluc* vector/pIB-*Rluc* vector (without 3′UTR of *hachi*) to optimize transfection regimen.

Quantitation of *miR-24* in *H. armigera* larvae. The expression level of *miR-24* in 5th instar larvae (1–5 day old) was estimated using QuantiMir RT kit Small RNA Quantitation system (SBI System Biosciences, USA). Briefly, cDNA pool of anchor-tailed miRNAs (QuantiMir cDNA) was prepared from midgut total RNA as per the manufacturer's guidelines. Real-Time qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) in StepOne™ Real-Time PCR System (Applied Biosystems, USA) and QuantiMir cDNA as template. The *miR-24* transcript was amplified using primers (*miR-24* For: 5′-TGAACTGAGGATCAACCCG-3′) and 3′ universal reverse primer (3′URP). The amount of total RNA was normalised to *β-actin* transcript using *β-actin* forward and reverse primers (mentioned earlier).

Each 30 μl reaction mixture contained 1 μl of diluted QuantiMir cDNA, 2X Power SYBR Green PCR Master Mix, 1 μl of *miR-24* For (10 μM) and 0.5 μl of 3′URP (10 μM) and RNAse-free water. Cycling and data accumulation conditions included an initial heating step at 50°C for 2 min, an initial activation step at 95°C for 10 min followed by 40 cycles each of denaturation (94°C, 15 sec), annealing and extension (50°C, 30 sec). The final step included gradual temperature increase from 60°C to 95°C at the rate of 0.3°C/10 sec to enable melt-curve data collection. A non-template control (NTC) was run with every assay. The threshold cycles (C_T) were recorded for each amplicon (*miR-24* and *β-actin*) and the difference between the C_T (i.e ΔC_T) was determined. The relative abundance was calculated using Comparative C_T method using the formula $2^{-\Delta\Delta C_T}$[26].

Force-feeding of *miR-24*. *H. armigera* larvae (4th instar, two-day old) were force-fed with 0.02 nmoles of synthetic *miR-24* (5′-UGAACUGAGGAUCAACCCG-3′) (Dharmacon, USA) using a microinjector (KPS210, KD scientific, USA). Control larvae were force-fed with Ringer's buffer while scrambled synthetic *miR-24* (5′-UAGGCGACUAUAACCCGGA-3′) force-fed larvae served as non-specific control. Each experiment was repeated thrice with 20 larvae per treatment. The chitinase transcript level in midgut tissue of both control and treated larvae was quantified by Real-Time qPCR.
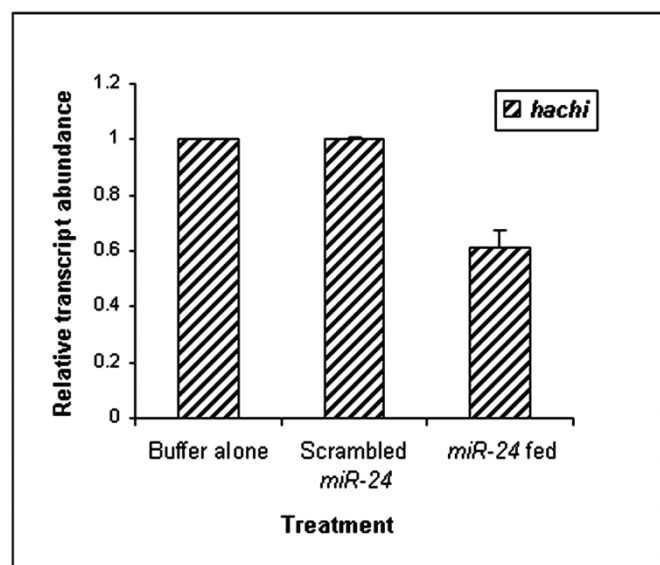
Figure 6 | Transcript abundance of *hachi* in *miR-24* fed *H. armigera* larvae. The *H. armigera* larvae (4th instar, two-day old) were force-fed with synthetic *miR-24* (0.02 nmoles) and midgut tissue was dissected 24 hrs post-feeding. Total RNA was isolated from the midgut tissue of *miR-24* fed (treated), scrambled *miR-24* fed (non-specific control) and buffer-fed (control) larvae. Chitinase transcript was examined by Real-time qPCR using *β-actin* as internal control. Expression levels of *hachi* were measured using Comparative C_T method. A non-template control was run with every experiment. Representative data (mean ±S.D.) from three independent experiments is shown.

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Author contributions

Additional information